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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

The central hypothesis we are addressing is that inhibition of mammary carcinogenesis by n-3 polyunsaturated fatty acids(PUFAs)can be accounted for by their inhibitory effect on the cholesterol biosynthesis (mevalonate) pathway. In Task 1, we have shown that the decrease in mammary gland HMG-CoA redustase seen in LDL-R -/- mice compared to +/+ mice fed a 7% n-6 diet persists when animals are are fed a 20% n-6 PUFA diet. The inherent poor susceptibility of C57Bl mice to chemically induced mammary carcinogenesis, however, precluded further work on this model. In Task 2, we have shown that exogenous mevalonate delivered to nude mice via osmotic mini-pumps promoted the growth of MDA-MB-435 human breast cancer cells, a result that supports our hypothesis. In Task 3, we have optimized conditions for the growth and treatment of MDA-MB-435 and MCF-10A cells and shown that long chain n-3 and n-6 PUFAs have differential effects on HMG-CoA reductase activity in at least one cancer cell line compared to normal mammary epithelial cells.Our work will provide a basis for understanding the protective effects of n-3 PUFAs and perhaps other dietary factors on breast cancer development and may lead to mechanism-based strategies for the prevention of breast cancer.

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INTRODUCTION

Consumption of fish oils rich in n-3 polyunsaturated fatty acids (PUFAs) is associated with a decreased risk of breast cancer in women [1-3]. These fatty acids also inhibit the development of chemically induced, transplanted and spontaneous mammary tumors in rodents [4-6]. The molecular mechanism of this inhibition, however, has not been elucidated. We have shown that dietary n-3 PUFAs down regulate 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase while increasing the levels of low-density lipoprotein receptors (LDL-R) in rat mammary glands [7]. HMG-CoA reductase catalyzes the formation of mevalonate that is required by mammalian cells for entry into the S phase of the cell cycle and has been postulated to play a role in cancer development [8-10]. This enzyme is also rate limiting in cholesterol biosynthesis and is down regulated when intracellular cholesterol levels rise [11]. Thus, the induction of LDL-R by n-3 PUFAs that we have observed may mediate the down regulation of HMG-CoA reductase by internalizing cholesterol-rich LDL particles. We hypothesize that inhibition of mammary carcinogenesis by n-3 PUFAs can be accounted for by their inhibitory effect on the mevalonate pathway. The objective of this research is to test this hypothesis in rodent models, and examine whether a similar mechanism could be operative in human breast cancer development.

BODY

Task 1. To determine the effects of dietary n-3 PUFAs on mammary tumorigenesis in LDL-R +/+ and LDL-R -/- mice (months 1-24).

The first objective of Task 1 was to produce mammary tumors in LDL-R +/+ and LDL-R -/- mice fed diets containing n-3 or n-6 PUFAs. Since writing the grant application, we

completed a series of experiments in which we investigated the regulation of mevalonate synthesis in LDL-R -/- mice fed n-3 or n-6 PUFAs [12]. Our results showed that dietary PUFAs and deletion of the LDL-R had independent effects on hepatic and mammary gland HMG-CoA reductase and serum lipids and we observed a significant diet-gene interaction. The effects of PUFAs on HMG-CoA reductase in the mammary gland, but not the liver, were mediated by the LDL-R. We also observed that differences in HMG-CoA reductase and serum LDL-cholesterol, high density lipoprotein cholesterol and triglycerides between -/- and +/+ mice were dependent on whether mice were fed n-3 or n-6 PUFA.

In view of the differences in lipid metabolism that we observed in -/- and +/+ mice fed the different diets, we decided to make a few changes in the design of the tumorigenesis experiment of Task 1. We had proposed to use dimethylbenz(a)anthacene (DMBA) as the initiating agent. DMBA is typically given *i.g.* dissolved in corn oil, and its absorption is mediated by bile acids [13]. As a lipid soluble compound, DMBA is transported from the liver to target tissues as a component of blood lipoproteins. We reasoned, therefore, that the difference in serum lipids between LDL-R +/+ and -/- mice would likely lead to differences in the pharmacokinetics of DMBA between the 2 types of mice. Thus, we decided that we must use the water soluble iniatiating agent methylnitrosourea (MNU) instead of DMBA to prevent the potentially confounding effect which a difference in carcinogen uptake and delivery between groups might have on initiation.

MNU initiates mammary tumorigenesis in mice, but a potential problem can be the development of lymphomas and ovarian tumors before mammary tumors have had the time to develop [14]. For this reason, mice are usually treated with hormones in mammary tumorigenesis experiments to reduce the latency and promote the growth of mammary tumors [14-16]. Indeed,

the background strain of the LDL-R -/- mice, C57Bl, is poorly susceptible to chemically-induced carcinogenesis [14], and we felt it would be necessary to treat these animals in some way to promote mammary tumor development. We chose, therefore, to treat all of the mice with the synthetic hormone medroxyprogesterone acetate (MPA) that has previously been shown to promote mammary tumorigenesis in the mouse [15, 16].

Work we performed to investigate the effects of n-3 and n-6 PUFAs feeding on mammary HMG-CoA reductase activity in LDL-R -/- versus wildtype mice was done using a 7% fat diet [7]. However, as we indicated in the grant application, Ip et al [17] have shown that diets must contain at least 4% linoleic acid to ensure adequate levels of this essential fatty acid for maximal mammary tumor growth. While the 7% safflower oil diet we used previously contains adequate linoleic acid, the 7% fat menhaden oil diet would be deficient, and may, therefore, artificially limit mammary tumor growth independent of any specific inhibitory effects of the individual PUFAs. To ensure adequate levels of linoleic acid in any future mammary tumorigenesis studies conducted, we have reformulated the test (n-3) and control (n-6) diets as follows: the 20% fat n-3 diet – 12% menhaden oil, 6% safflower oil, 2% soybean oil; the 20% fat n-6 diet – 18% safflower oil, 2% soybean oil.

In view of these changes from the diets we previously used, we decided it was necessary to perform a preliminary experiment to ensure that the decrease in mammary HMG-CoA reductase activity seen in LDL-R -/- mice compared to wildtype mice fed the 7% n-6 PUFA diet persisted when animals were fed the 20% n-6 PUFA diet. Briefly, 4 LDL-R -/- and 4 LDL-R +/+ mice were fed the 20% fat n-6 diet for one week. Animals were sacrificed by CO_2 asphyxiation and microsomes were isolated from mammary and liver tissues. Both mammary and hepatic HMG-CoA reductase activity levels were siginificantly lower in LDL-R -/- animals versus

wildtype (see Figs 1a and 1b), and were, indeed, comparable to those we observed in mice fed the 7% n-6 diet [12].

In view of the issues discussed above, we decided it would be prudent to carry out a preliminary experiment to determine the feasibility of the full experiment described in Task 1. We decided to focus on the hypothesis that the lower HMG-CoA reductase activity in LDL-R -/- mice compared to +/+ mice (see Fig 1b) will lead to a lower mammary tumor incidence when the mice are fed diets rich in n-6 PUFAs.

Thirty female LDL-R knockout mice and 30 C57Bl wildtype controls were purchased from Jackson Labs at 5 weeks of age and acclimatized on an AIN-93G standard diet for one week. At 6 weeks of age animals were injected *s.c.* with 40 mg of slow-release medroxyprogesterone acetate (MPA) (Depo-Provera®) in the interscapular area. At 8 weeks of age, animals received a single dose of 50mg/kg MNU. One week post-MNU all animals were switched to the 20% n-6 diet for the duration of the experiment. Animals were weighed biweekly and palpated for the presence of mammary tumors weekly. Animals were given fresh food biweekly, and consumption per cage was recorded.

Throughout the study there was no significant difference in food consumption or weights between the two groups. Approximately one month post-initiation, some animals unexpectedly began "barbering", a process of excessive grooming that leads at first to superficial hair loss, but if continued aggressively, to eventual skin damage and tissue destruction in the area affected. By ~2.5 months post-MNU, we sacrificed some animals that had significant damage to the skin and underlying connective tissue, or that had lost weight. Steps were taken to minimize barbering activity & damage – barbered animals were separated to prevent possible barbering by the dominant female in the cage, and animals with skin lesions were treated with varitone to prevent

infection, and to lessen damage by reducing irritation in the area. Sacrificed animals were necropsied, and samples of mammary, liver, spleen, kidney, and ovaries were taken for histopathology. Several mice had grossly enlarged spleens, and all had marked anaemia. Because of the barbering activity, we decided to terminate the experiment at 6 months post-initiation. At this time, all mice were killed and samples of mammary gland, ovaries, liver, kidney, and spleen were placed in 10% buffered formalin for paraffin embedding. No palpable, or grossly visible mammary tumors were evident in either group. Slides prepared from mammary glands of mice indicated the growth of malignant lymphomas in mammary fat pads in most animals, but no mammary tumors were apparent. No differences in lymphoma incidence between LDL-R -/- and wildtype mice were found. The inherent poor susceptibility of C57Bl mice to chemically induced mammary carcinogenesis indicates caution in proceeding with further work on task 1, or in studies utilizing similar models.

Task 2 To determine whether transgenic mice that overexpress the LDL-R in the mammary gland develop fewer tumors than wild-type controls.

Transgenic mice are currently created in two predominant background strains – FVB and C57Bl. Both of these strains are poorly susceptible to the induction of chemically-induced mammary tumors. Given our experience with chemical carcinogenesis in C57Bl mice (see Task 1), and that other, more susceptible strains of mice are unavailable for generation of transgenic animals, we realized that it would be prudent to pursue another approach to test our central research hypothesis. The principle aim of this work has been to establish definitively whether mevalonate plays a role in mammary carcinogenesis. To this end we have examined two possible alternative routes of investigation. The first involved the use of the well-established rat chemical

carcinogenesis model, which reproducibly yields a high incidence of mammary tumors (>80% in our hands) with short latency (<26weeks). We proposed to investigate the effects of exogenous mevalonate on the growth of chemically-induced rat mammary tumors. An abundant exogenous mevalonate supply available to growing transformed cells of the mammary gland would down-regulate HMG-CoA reductase activity, while still allowing cells to produce all of the down-stream mevalonate-derived metabolites. This model would be equally as useful for us as the transgenic LDL-R mouse and would avoid the problems associated with the mouse being resistant to chemical carcinogenesis. However, it was first necessary to address the feasibility issue of delivery of mevalonate to target tissues. To determine a method of mevalonate delivery to rat mammary glands, several small preliminary studies were conducted, as follows:

Study 1: Ten female Sprague Dawley rats were fed a diets containing either 0 or 1% mevalonate (wt/wt) for a period of 1 week. Animals were then sacrificed and liver and mammary gland tissues were assayed for HMG-CoA reductase activity. Enzyme activity down-regulation was taken as evidence of product-feedback inhibition, indicating delivery of mevalonate to target cells. While hepatic HMG-CoA reductase activity decreased substantially (Fig 2a), mammary gland reductase activity did not change significantly (Fig 2b). Taken together, this likely indicates rapid hepatic clearance of mevalonate during first pass through the liver following intestinal absorption, and therefore inadequate delivery of mevalonate to the mammary gland.

Study 2: As an alternative route to oral administration of mevalonate, we assessed the ability of mini-osmotic pumps to deliver a physiological dose of mevalonate to the rat mammary gland. As in study 1, effective delivery of mevalonate was assessed by measuring feedback inhibition of HMG-CoA reductase. Briefly, 10 female SD rats, age 12 weeks, were randomized to two groups, and surgically implanted *s.c.* with a 200 uL mini-osmotic pump (flow rate of 0.25uL/hour, and

pumping 'life' 28 days) containing either 1mg/ul mevalonate, or isotonic saline. Rats were sacrificed two weeks after pump implants, and HMG-CoA reductase activity was assessed. Results again indicated no significant difference in mammary reductase activity between groups (see Fig 3), indicating insufficient delivery of mevalonate to mammary cells by 200 uL pumps. Study 3: To assess whether a greater dose of mevalonate to rats would reach target cells of the mammary gland, 10 rats were implanted *s.c.* with 2 mL mini-osmotic pumps capable of releasing a ten-fold greater dose of mevalonate than that used in study 2 (flow rate 2.5uL/hour). However, even at this dose, mevalonate released *s.c.* failed to reach cells of the mammary gland (see Fig 4).

At this time, we began to explore a second alternative avenue of investigation to address the role of the mevalonate pathway in mammary carcinogenesis. Athymic nude mice are able to accept xenografts, including an injection of human breast cancer cells, which subsequently grow to form a palpable tumor. Tumor growth in nude mice has been shown to be affected by treatments administered to the host mouse, including drug treatments and alterations in diets [18]. We proposed to perform an experiment wherein the ability of exogenous mevalonate to modulate the growth of human breast cancer cells inoculated into the mammary fat pad of nude mice would be assessed. A feasibility study was performed in Balb/c mice, the background strain for the nude mouse, to determine a mode of delivery of exogenous mevalonate to the mammary gland. *S.c.* implantation of mini-osmotic pumps (flow rate 0.25uL/h) containing mevalonate significantly down-regulated mammary HMG-CoA reductase activity (Fig 5) indicating effective delivery of mevalonate to mammary glands of mice.

To determine the effects of increased exogenous mevalonate on the growth of human breast cancer cells in nude mice, the following experiment was performed. 60 female athymic

nude mice, age 8 weeks, were inoculated in the right thoracic mammary fat pad with 10^6 highly malignant, metastatic MDA-MB-435 human breast cancer cells. One week following inoculation, animals were surgically implanted *s.c.* in the inter-scapular area with a mini-osmotic pump containing either 1g/L mevalonate or isotonic saline. Animals were fed an AIN 93G control diet thoughout the experiment. Beginning 2 weeks after pump implantation, when wounds had healed, animals were weighed and palpated weekly for tumors. Palpable tumors were measured using vernier calipers. At monthly intervals, spent pumps were removed surgically, and replaced with fresh pumps. At week 13, animals were sacrificed and tumors were weighed and measured. Sections of mammary gland, tumor, and perfused lungs were preserved in 10% buffered formalin.

Tumors in both groups grew at similar rates until the time period following the third pump replacement. From approximately 9 weeks onward, tumors in mice treated with mevalonate grew at a significantly greater rate than controls. By week 13, tumors in mevalonate treated animals had a greater surface area (Fig 6a) and weight (Fig 6b) versus controls. We are currently performing immunohistochemistry to determine whether increases in tumor size resulting from mevalonate treatment are associated with evidence of enhanced angiogenesis in tumor sections. Our results would indicate that growth of mammary tumors in this model is limited by the concentration of mevalonate, a result that supports our hypothesis that this metabolite plays a key role in controlling the rate of cell proliferation.

Task 3   To determine the extent to which growth inhibition by n-3 PUFAs is reversed by mevalonate in normal and neoplastic breast epithelial cells.

In initial experiments we have proceeded with optimization of cell culture conditions. The issue of ensuring that the non-polar test fatty acids are adequately solubilized in aqueous media has prompted us to experiment with varying culture conditions, including different levels of ethanol (up to 1%) and albumin (up to 2g/L). Reported conditions in the literature vary considerably, even for commonly used cell lines, and so we wanted to ensure that culture conditions achieved optimal solubilization of fatty acids, while maintaining favorable growth conditions and minimizing toxicity of vehicles used. Our current protocol involves extensive mixing and pre-incubation of fatty acids with albumin (final concentration in media 2g/L) to allow for maximal binding to the protein carrier. Final ethanol concentrations are minimized, with a maximum level in media of 0.05%. Serum free conditions are used for experimental treatment of all cells lines except MCF-7, which requires low-serum medim (0.5% fetal bovine serum) for growth.

Our work involves the use of four cell lines, including the estrogen-independent breast cancer lines MDA-MB-435 and MDA-MB-231, the estrogen-dependent line MCF-7, and the normal mammary epithelial cell line MCF-10A. We have partially completed characterization of these lines necessary for perfomance of growth experiments. MDA-MB-435 and MCF-10A cell lines both proliferate rapidly and show responses to treatments within as little as three days. Treatment of these two cell lines with 30 μM eicosapentaenoic acid (EPA) for 96 hours (4 days) causes an approximate 40 to 50% inhibition of proliferation (see Figs 7a and 7b). Conversely, treatment with 30 μM linoleic acid (LA) causes a significant increase in proliferation rate in MDA-MB-435 cells, but no change in MCF-10A cells. Mevalonate treatment of both cell lines, up to 500 μM, does not influence cell proliferation. Experiments determining the ability of

mevalonate to rescue EPA-induced inhibition of MDA-MB-435 and MCF-10A cells are currently under way.

In the slower growing MCF-7 and MDA-MB-231 cell lines, four days has been found to be inadequate to establish an effect of fatty acid treatments on proliferation. We are currently determining optimal fatty acid treatment levels during 6 day incubations.

For determination of HMG-CoA reductase activity, cells from each cell line were grown for 4 days in 75 dL flasks in treatment media containing 25 μM or 50 μM of the test fatty acids EPA and LA. As was found in the cell proliferation assays, 4 days was inadequate to effect any change in HMG-CoA reductase activity in MCF-7 and MDA-MB-231 cells. Longer treatment intervals are being tested. In accord with *in vivo* data from normal rodent mammary gland, exposure of MCF-10A normal human mammary epithelial cells to EPA resulted in a significant down regulation of HMG-CoA reductase activity versus cells treated with LA (Fig 8a). However, unexpectedly, treatment of MDA-MB-435 cells with 25 μM EPA significantly increased HMG-CoA reductase activity versus treatment with 25 μM LA (Fig 8b). Presently, we are in the process of replicating experiments to confirm our observations. Following complete optimization of cell culture conditions, work on LDL-R expression modulation by fatty acid treatments will commence.

KEY RESEARCH ACCOMPLISHMENTS

• Surgically implanted 200uL mini-osmotic pumps containing 1g/mL mevalonate were shown to deliver mevalonate to the mammary gland of mice. This model was not effective for mevalonate deliver in the rat, and a suitable model remains elusive.

• Exogenous mevalonate was demonstrated to promote the growth of tumors derived from MDA-MB-435 human breast cancer cells, growing in athymic nude mice.

• Optimal conditions for the growth and treatment of MDA-MB-435 and MCF-10A cells were established.

• Long chain n-3 and n-6 PUFAs were demonstrated to have differential effects on HMG-CoA reductase activity in at least one cancer cell line versus normal mammary epithelial cells.

REPORTABLE OUTCOMES

Meeting abstracts

1. Diet-gene effects in cancer development. Ottawa Life Sciences National Conference, December, 2000.

2. Diet-gene effects in cancer development. Canadian Federation of Biological Societies, June, 2001.

Personnel receiving pay from this award

Robin Duncan, Suying Lu

CONCLUSIONS

While technical limitations have diverted our use of transgenic and knockout mice in the chemical carcinogenesis studies we originally proposed, work involving the nude mouse-tumor cell inoculation model has been productive. In preliminary feasibility studies we determined that implanted mini-osmotic pumps could be used to deliver mevalonate to the mammary glands of mice, but not rats. This excluded the possibility of investigating the effects of mevalonate on rat mammary carcinogenesis, but allowed us to go ahead with our investigation of the effects of mevalonate on the growth of human breast cancer cells in mice. Tumors derived from highly

malignant, metastatic MDA-MB-435 human breast cancer cells grew more rapidly in nude mice implanted *s.c.* with mini-osmotic pumps containing mevalonate compared to controls, and had greater weights and volume at the end of the experiment (13 weeks). This indicates that growth of mammary tumors in this model is limited by the concentration of mevalonate, a result that supports our hypothesis that this metabolite plays a key role in controlling the rate of cell proliferation.

In normal rodent mammary tissue, a diet rich in n-3 PUFAs inhibits HMG-CoA reductase activity, thereby limiting endogenous mevalonate synthesis compared to a diet rich in n-6 PUFAs. To investigate whether n-3 PUFAs inhibit breast tumor cell proliferation by inhibiting endogenous mevalonate synthesis, we are currently performing cell culture work in which the ability of mevalonate to rescue inhibition of cell proliferation by n-3 PUFAs (EPA and DHA) is being ascertained in 3 human breast cancer cell lines, and 1 normal mammary epithelial line. We are also measuring HMG-CoA reductase activity in cell lines treated with n-3 and n-6 PUFAs. Preliminary data indicates that normal mammary epithelial cells (MCF-10A) in culture respond similarly to mammary cells *in vivo*, undergoing a down-regulation of HMG-CoA reductase activity in response to treatment with the n-3 PUFA EPA, compared to the n-6 PUFA LA. However, unexpectedly, EPA appears to dramatically induce HMG-CoA reductase activity in MDA-MB-435 cells compared to LA. The significance of this finding remains to be determined but could be of great value in our mechanistic studies.

Mevalonate is a key metabolite in all living cells, required for the synthesis of cholesterol, and hence membranes, and for the production of the isoprenoids which serve a myriad of growth regulatory functions. Indeed, mevalonate is required for DNA synthesis and cell proliferation. Several identified plant compounds, as well as fish oils, can inhibit mevalonate

synthesis in cells. Successful completion of our experiments will provide a basis for understanding the protective effects of n-3 PUFAs and, perhaps, other dietary factors on breast cancer development and may lead to mechanism-based strategies for the prevention of the disease.

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APPENDIX

Figures

Liver HMG-CoA Reductase Activity
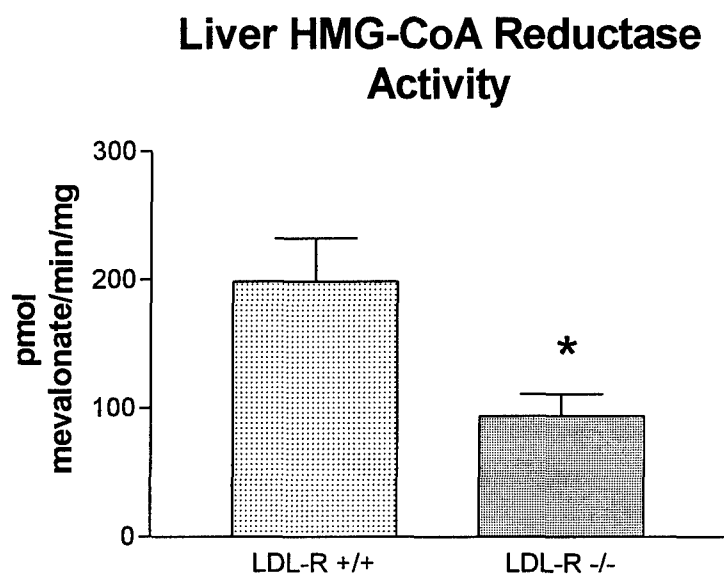
Figure 1a   Liver HMG-CoA reductase activity in LDL-R-/-
versus LDL-R +/+ mice fed a 20% fat diet rich in n-6 PUFAs
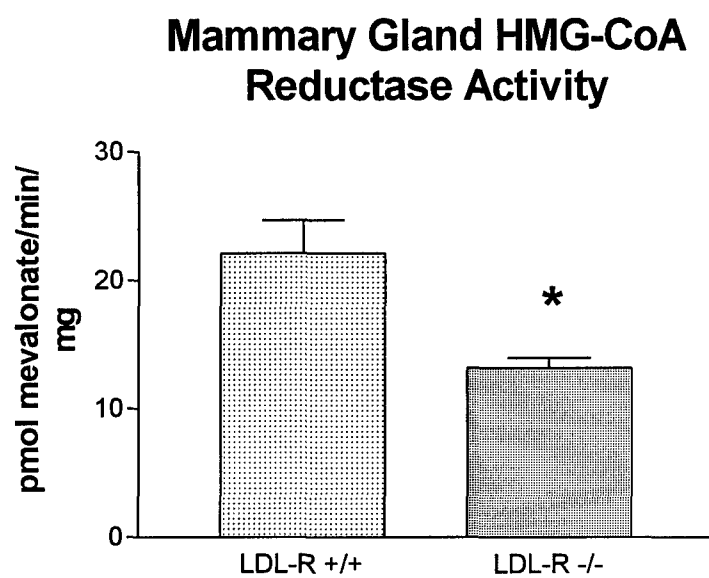(measured in pmol mevalonate produced per minute per mg
microsomal protein), p<0.05.

Mammary Gland HMG-CoA Reductase Activity

Figure 1b   Mammary HMG-CoA reductase activity in LDL-R-/-
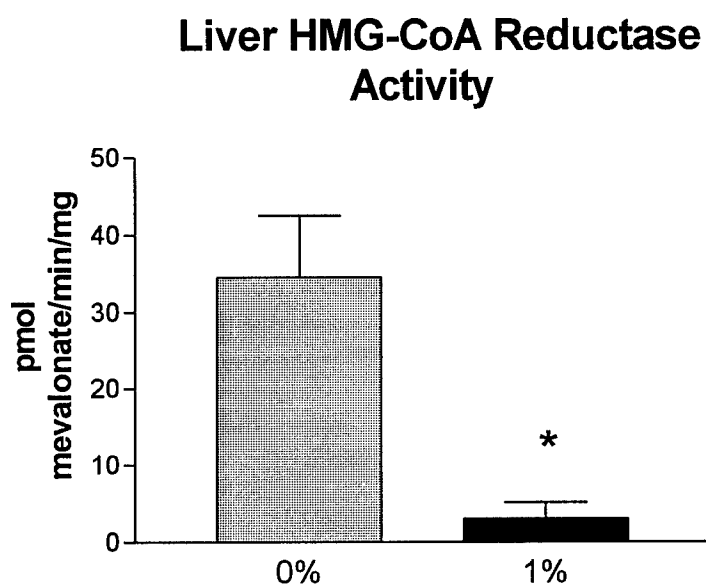versus LDL-R +/+ mice fed a 20% fat diet rich in n-6 PUFAs.* p<0.02.

Liver HMG-CoA Reductase Activity

Figure 2a   Liver HMG-CoA reductase activity in Sprague Dawley rats fed an AIN 93G control diet containing 0% or 1% mevalonate (wt/wt). $p < 0.02$

Mammary Gland HMG-CoA Reductase Activity
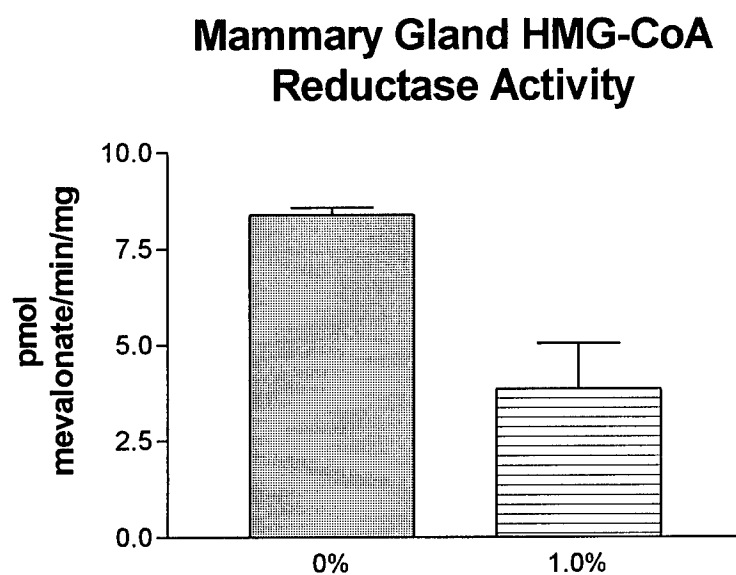
Figure 2b   Mammary HMG-CoA reductase activity in Sprague Dawley rats fed an AIN 93G control diet containing either 0% or 1% mevalonate (wt/wt).

Mammary Gland HMG-CoA reductase activity
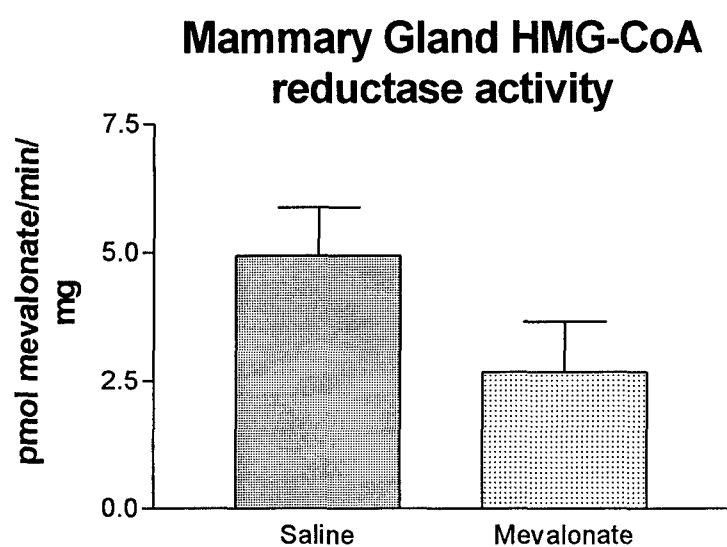
Figure 3   Mammary HMG-CoA reductase activity in rats implanted *s.c.* with a 200uL mini-osmotic pump delivering mevalonate or isotonic saline (0.25uL/h).

Mammary HMG-CoA Reductase Activity
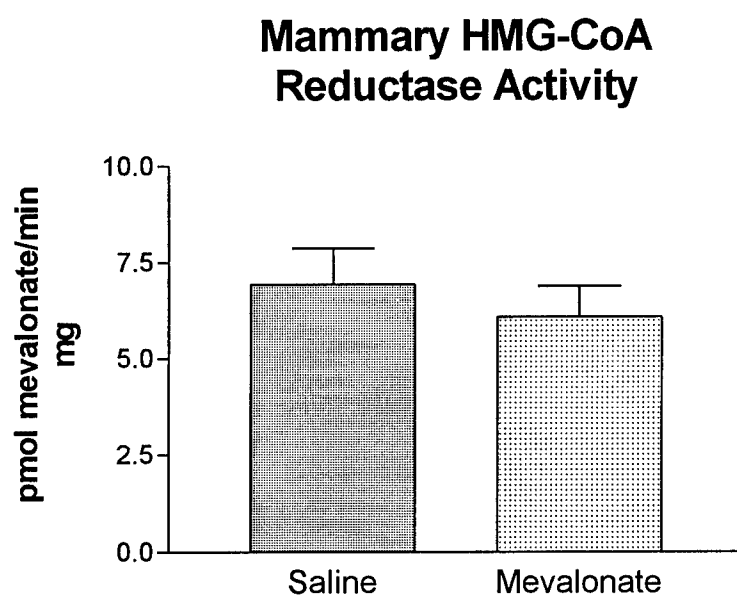
Figure 4   Mammary HMG-CoA reductase activity
in rats implanted *s.c.* with a 2mL mini-osmotic pump
delivering mevalonate or isotonic saline (2.5uL/hr).

Mammary Gland HMG-CoA reductase activity
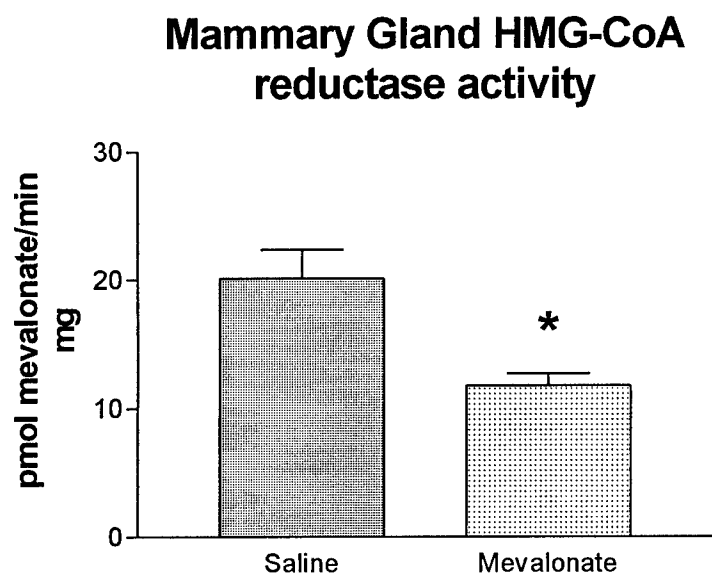
Figure 5   Mammary HMG-CoA reductase activity in Balb/c mice implanted s.c. with 200uL mini-osmotic pumps containing mevalonate or isotonic saline (0.25uL/h).*p<0.005

Tumor Growth Rate
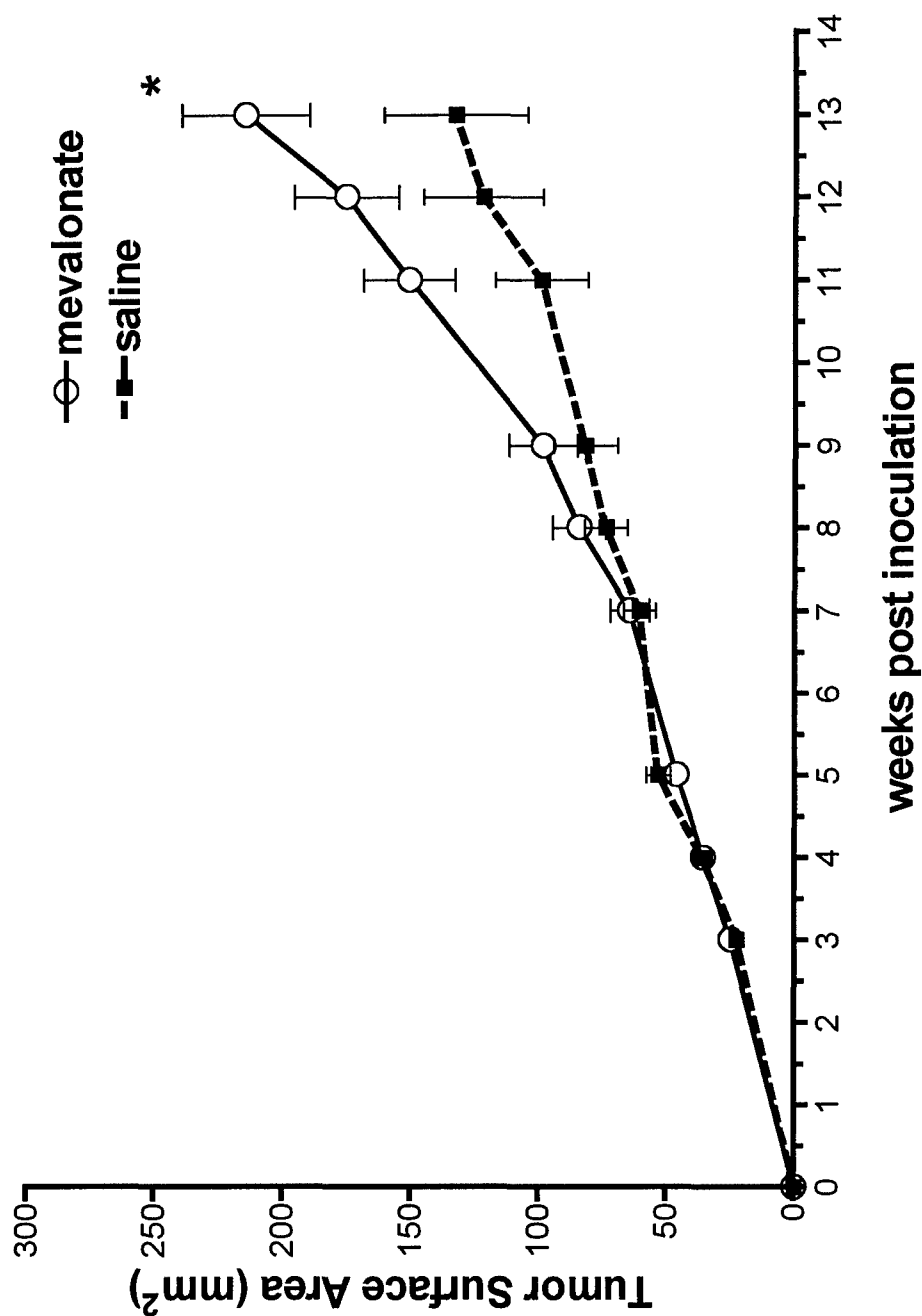
Figure 6a   Surface area of tumors derived from MDA-MB-435 human breast cancer cells growing in athymic nude mice treated with either mevalonate or isotonic saline delivered via mini-osmotic pumps. *p<0.05
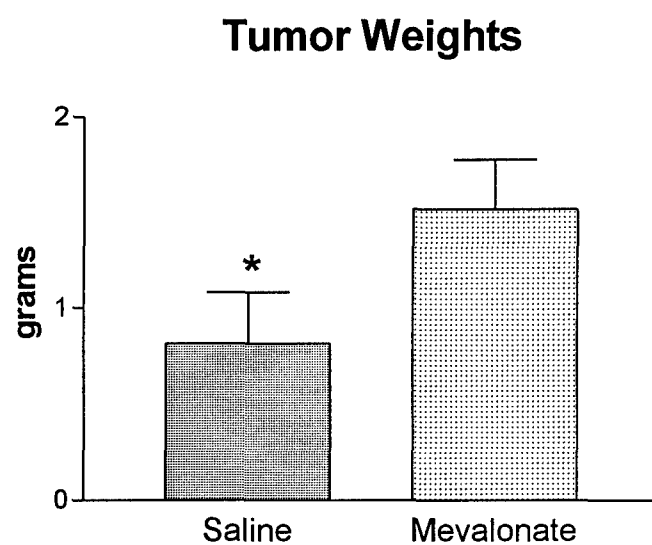
Tumor Weights

Figure 6b   Final weights of tumors derived from
MDA-MB-435 human breast cancer cells grown in
nude mice treated with mevalonate or saline via *s.c.*
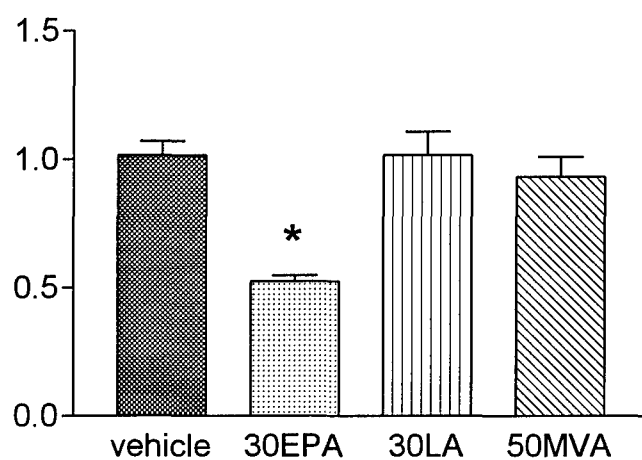implaned mini-osmotic pumps. p<0.05

MCF-10A Cell Proliferation

Figure 7a   Proliferation rate of MCF-10A
cells treated with 30uM EPA, 30uM LA or 50uM
mevalonate measured relative to control
(vehicle) levels. *p<0.01
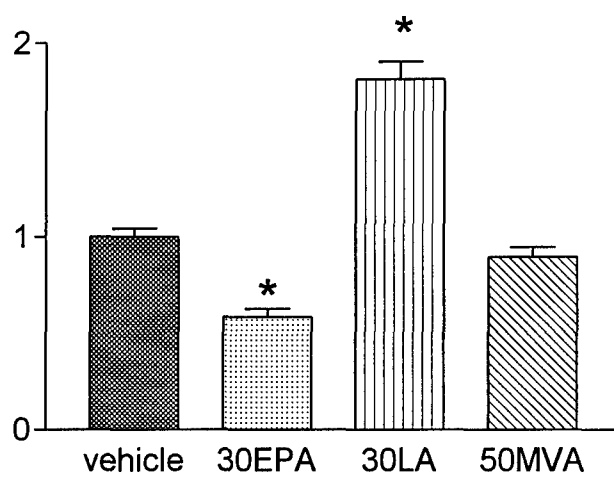
MDA-MB-435 Cell
Proliferation

Figure 7b   Proliferation rate of MDA-MB-435
cells treated with 30uM EPA, 30uM LA or 50uM
mevalonate relative to control (vehicle) levels.
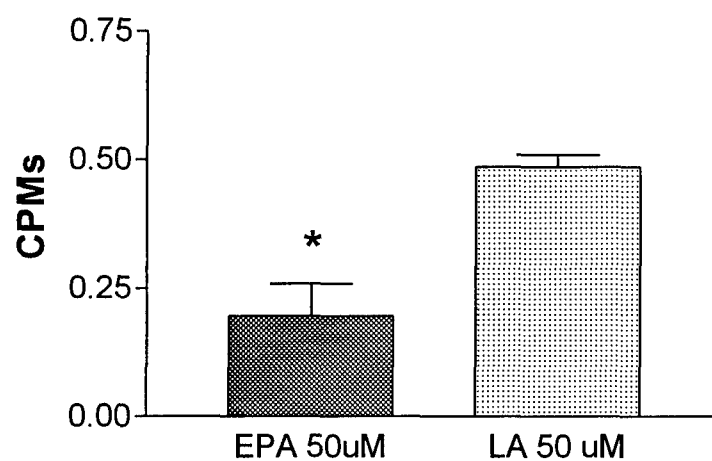*p<0.01

MCF-10A HMG-CoA Reductase Activity

Figure 8a   HMG-CoA reductase activity of
MCF-10A cells treated with 50uM EPA or 50uM
LA (counts per minute measured using Instant
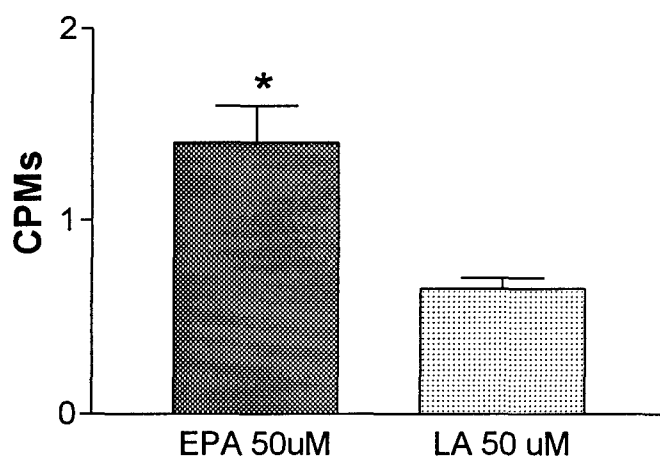Imager). *p=0.005

MDA-MB-435 HMG-CoA Reductase Activity

Figure 8b   HMG-CoA reductase activity of MDA-MB-435 cells treated with 50uM EPA or 50uM LA (counts per minute measured using Instant Imager). *p<0.01